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Genetic analysis shows that *Rubus vikensis* is a distinct species with a disjunct distribution

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Abstract

Rubus vikensis A. Pedersen ex G. Wendt (sect. *Corylifolii*) was recently described from a restricted area in north western Scania, Sweden. In this investigation, I show that the same species occurs also on the Onsala peninsula in northern Halland and on a single locality in the middle of Halland. It has 35 chromosomes in all parts of the distribution area. Moreover, I show by random amplified polymorphic DNA (RAPD) analysis that *R. vikensis* is a distinct and well-defined species, clearly separated from the morphologically similar species *R. wahlbergii*, with which it shares the chromosome number.

Key Words: *Rubus* sect. *Corylifolii*, flow cytometry, RAPD

Introduction

Rubus sect. *Corylifolii* constitutes a large and diverse group of blackberries that is assumed to have arisen through hybridisation between true blackberries (*R.* sect. *Rubus*) and dewberry (*R. caesius* L.). The variation is preserved through the formation of fruits without fertilisation, apomixis (Gustafsson 1943). As an effect, a large number of closely related taxa have arisen. Currently, almost 30 indigenous *Corylifolii* species are known from in Sweden, but the number is steadily increasing because new species are found or described.

Recently, a new *Corylifolii* species, *R. vikensis* A. Pedersen ex G. Wendt, was described by Wendt (2008). It occurs on almost 60 localities in north western Scania (southernmost Sweden), especially around the city of Helsingborg. It is a vigorous plant, with heart-like terminal leaflets, which are white tomentose beneath, like *R.* wahlbergii Arrh. It differs from that species by a large number of glands in both the inflorescence and on the turions, more elongated terminal leaflets, larger and paler flowers (pink only as young), and more slender prickles. However, it has more often been confused with *R. tiliaster* H. E. Weber, with which it shares the shape of the terminal leaflet, although the anthers are glabrous.

During my inventory of the *Corylifolii* species in the province of Halland (Ryde 2009), a form was found that shares most macroscopic characters with *R. vikensis* (Figure 1). It occurs on the Onsala peninsula, 140 km north of the known localities of *R. vikensis* in Scania. A similar form was also found on a single locality in Trönninge, south of Halmstad, halfway between the two groups of localities. It has been collected earlier from both areas, but it was incorrectly determined to *R. norvegicus* H. E. Weber & A. Pedersen (Georgson et al. 1997, Lye & Pedersen 1994). However, considering the great morphological variation within *Rubus* species, it is hard to decide whether the three populations belong to the same species or if they are the result of convergent speciation.

In this study, I estimate the chromosome number and use random amplified polymorphic DNA (RAPD) analysis to study the genetic relation between these forms. RAPD produces a multi-locus band profile of dominantly inherited DNA fragments. It has proven useful to differentiate between genotypes, discriminate between apomictically and sexually derived offspring, detect recent hybridization in nature, estimate diversity both within and between populations, and provide phenetic analyses of similarities and dissimilarities among the different species of genus *Rubus* (Graham & McNicol 1995, Graham et al. 1997, Werlemark & Nybom 1999, Pamfil et al. 2000, Graham et al. 2003, Weber 2003, Stafne et al. 2003, Randell et al. 2004, Coyner et al. 2008, JinBo et al. 2008, YuanYuan et al. 2009, HanWu et al. 2009).

Methods

RAPD analysis

Leaves were sampled in June 2008 from five localities of *R. vikensis*: two from northern Halland, two from Scania, and from the locality in Trönninge (see Table 1), including a duplicate sample from locality 2. For comparison, material of *R. wahlbergii* from one locality in Halland and one in Scania was also sampled. The leaves were kept at –80°C until used. One or two leaves (30–60 mg fresh weight) were crushed in an Eppendorf tube with a glass rod in 400 µl of extraction buffer, provided with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). All further steps of the extraction procedure followed the manufacturer's instructions and the samples were stored in 1· TAE buffer and kept at +4° C until amplifications.

The PCR amplifications were carried out in a reaction mixture containing approximately 20 ng of DNA, 1· reaction buffer IV (Advanced Biotechnologies), 1.5 mM $MgCl_2$, 0.6 µM primer (Operon Tech. Inc.) , 0.2 mM PCR Nucleotide MIX (Roche) and 1.0 unit Taq DNA

polymerase (Advanced Biotechnologies). The mixture was filled up with ultra pure water to 25 µl. Amplifications were carried out in a thermocycler (MJ Research) under the following conditions: 5 minutes initial denaturation at 94°C, amplification for 40 cycles of 1 min at 94° C, 1 min at 36° C, and 2 min at 72° C, followed by 7 min extension time at 72° C. The amplification products were then separated by electrophoresis in a 1.8% agarose gel in 1· TAE buffer containing 0.5 µg/ml ethidium bromide, and were subsequently photographed under UV light.

The pictures were scored manually for presence and absence of bands. As there were so few samples, no attempt was made to find primers that gave the most polymorphic bands. Instead, all 17 primers from Operon Technologies were used and the primers with readable and reproducible bands were used (Table 2).

To analyse the relationships between the samples, multidimensional scaling analysis (MDS) was used. This analysis provides a visual representation of non-hierarchical proximities (similarities or dissimilarities) among the samples in the form of a two-dimensional plot. The distances between the samples are correlated with the actual distances in the plot. The computation was performed with the SPSS statistical program, version 13.0.0. All parts of the RAPD analysis were performed at SLU Balsgård.

Flow cytometry

The samples were also subjected to flow cytometry analysis by Plant Cytometry Services, JG Schijndel, The Netherlands (http://www.PlantCytometry.nl). The leaves were chopped in ice-cold buffer with DAPI (Arumuganathan & Earle 1991). Flow cytometry was performed on a CyFlow ML (Partec GmbH, Münster, Germany) using *Lactuca sativa* (iceberg lettuce) as internal standard. Duplicate samples from all forms of *Corylifolii* occurring in Halland were also included in this investigation, *R. camptostachys* G. Braun*, R. eluxatus* Neum.*, R. fasciculatus* P. J. Müll.*, R. gothicus* Frid. & Gelert ex E. H. L. Krause*, R. hallandicus* Neum.*, R. lagerbergii* Lindeb.*, R. mortensenii* Frid. & Gelert ex E. H. L. Krause*,* and *R. norvegicus*. All of them have known chromosome numbers (they have $2n = 28$, except *R.* lagerbergii with $2n = 35$; Gustafsson 1943). A sample of the cultivar *R.* 'Bedford Giant' with $2n = 42$ was also included. Together, they provided an excellent calibration line for the estimation of the chromosome numbers of the samples of *R. vikensis* and *R. wahlbergii*: 104 times the ratio between the sample and the internal standard. The mean absolute deviation between the calibration line and the samples with known chromosome numbers was 0.5, with a maximum of 2.0.

Results

RAPD analysis

In total, 17 primers were tested and 13 of these gave clear and polymorphic bands (Table 2). 69 polymorphic bands were achieved, i.e. 5.3 bands/primer. Even without the statistical analysis, it was clear that the five samples of *R. vikensis* (the two duplicates gave identical results) were very similar, whereas the samples of *R. wahlbergii* were more variable. Within *R. vikensis*, there was a difference in 3–8 bands among the samples, whereas the two species differed by at least 36 bands. The two samples of *R. wahlbergii* differed by 18 bands. The results are succinctly presented in the two-dimensional MDS plot in Figure 2, which shows a good separation between the two groups. The proportion of variance explained by the two axes was 0.998, with Kruskal's stress of 0.026, which indicates that the data is well represented by the two-dimensional plot.

In conclusion, the RAPD analysis clearly shows that *R. vikensis* with localities in Scania,

as well as in Trönninge, Onsala, and Vallda in Halland, is a homogeneous species, well separated from *R. wahlbergii*, and actually with a much smaller intraspecific variation than the latter species (which according to morphological characters is an unusually variable species within sect. *Corylifolii*).

Chromosome numbers

The chromosome numbers of the samples of *R. vikensis* were determined by flow cytometry to $2n = 35\pm1$ (Table 1). This number has also been obtained before for *R. vikensis* from Scania (A. Oredsson, pers. comm.). The same number was observed for *R. wahlbergii* from the localities in both Scania and Halland, and it has also been reported before (Gustafsson 1943). This chromosome number is unusual in sect. *Corylifolii;* besides *R. wahlbergii* and *vikensis,* it is in Sweden found only for *R. lagerbergii, R.* pruinosus Arrh., and *R. rosanthus* Lindeb. (Gustafsson 1943), as well as in hybrids with *R. idaeus* L. (Ryde 2009). Most other *Corylifolii* species have $2n = 28$, whereas *R. tiliaster* has $2n = 42$.

Discussion

The genetic results clearly show that *R. vikensis* is a well-defined and homogeneous species. They confirm the macroscopic observation that *R. vikensis* from Scania, and the two groups of localities in Halland are similar and well separated from similar species, such as *R. wahlbergii.* Thus, *R. vikensis* has a wide, but rather disjunct distribution (Figure 3), with one centre in north-western Scania, especially around the city of Helsingborg and along the coast up to the village of Viken, and another centre on the Onsala peninsula. In addition, there is an isolated locality around a marl pit in Trönninge, south of Halmstad. Unfortunately, the latter locality is threatened, being close to newly-built houses and severely pressed by reeds. The largest distance between the localities is almost 170 km, thus making it a regional species, according to system of Weber (distribution of 50–250 km across; Weber 1981). The largest distance among the Scanian localities is 33 km. A list of the Scanian localities has been published before (Wendt 2002), whereas the localities in Halland are given in Appendix 1.

In Onsala, I have found *R. vikensis* in 45 localities within only 2 km. In this restricted area around Vässingsö, Godhem, Runås, Knastås, and Röda Holmen on the southernmost part of the Onsala peninsula, it is the most common *Corylifolii* species, forming wide stands on the roadsides. In the same area, *R. wahlbergii* is frequent, and *R. gothicus, R. norvegicus* and *R. fasciculatus* are also found. In addition, *R. vikensis* occurs on a single locality in the village Buera in Vallda parish, 7.5 km to the north-west. There, it occurs on a roadside, with *R. gothicus* nearby.

R. vikensis has been collected several times from Halland before. The first collection seems to be from Trönninge by Neuman 1883 (Georgsson et al. 1997). It has also been collected from Godhem in Onsala by Lundberg 1934. Unfortunately, all these collections were incorrectly determined to *R. norvegicus* (Georgsson et al. 1997, Lye & Pedersen 1994). Thus, *R. vikensis* from north-western Scania was originally mistaken as *R. tiliaster* (which occurs in this area), whereas specimens from Halland were called *R. norvegicus*. This shows that it tempting to determine blackberries according to where they was collected. Before I realised that this form was identical to *R. vikensis*, I tentatively called it "*R. godhemensis*" in my *Corylifolii* home pages (Ryde 2005), on some locally distributed determination keys, and on redetermined exsiccates in the public Swedish botanical museums.

Disjunct distributions, like that *of R. vikensis*, are not rare among the blackberries. For example, *R. sprengelii* Whe. occurs in both south eastern Scania and southern Bohuslän, whereas *R. lagerbergii* occurs in north-western Scania and around the city of Göteborg, including the northernmost part of Halland, with no localities in between, although it is quite common in both areas. This may be the effect of rare occurrences of long-range dispersion,

e.g. with the help of migratory birds. It is possible that *R. vikensis* occurs also outside Scania and Halland; it should be looked for in adjacent parts of Sweden and Denmark.

The present study also shows that RAPD provides a powerful method to discriminate between different species, also among the *Corylifolii* (I am not aware of any RAPD study of *Corylifolii* before). The results in Figure 2 shows that there are some differences in intraspecific variation among different *Corylifolii*: *R. vikensis* is a very uniform species, whereas *R. wahlbergii* shows a much larger diversity. This is also confirmed morphologically, although the two samples in this investigation appeared similar and quite typical. It is also notable that the *Corylifolii* species seem to give a smaller variations (a lower number of polymorphic bands per primer) than species from other sections of *Rubus*: We observe only 5 polymorphic bands per primer on average, whereas previous studies have shown 12–22 (YuanYan et al. 2009, HanWu et al. 2009). This probably reflects that the *Corylifolii* are obligate apomictic, whereas many of the previously studied species are sexual or facultatively apomictic.

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Table 1. List of examined material. Double samples were taken from locality 2. $2n_{est}$ is the chromosome number estimated by flow cytometry.

#	Species	Province	Parish	Locality	Coordinates		$2n_{est}$
1	*R. vikensis*	Halland	Vallda	Hästakärrsvägen N of Buera	6374559	1267609	35
2	*R. vikensis*	Halland	Onsala	Crossing between Godhemvägen and Vässingsövägen	6367415	1270872	34
3	*R. vikensis*	Halland	Trönninge[1]	Marl pit, 450 m SW of Trönninge church	6280569	1323531	35
4	*R. vikensis*	Scania	Helsingborg	Fredriksdal at Lägervägen	1307700	6218300	35
5	*R. vikensis*	Scania	Viken	550 m SW of Viken church	6228631	1299842	34
6	*R. wahlbergii*	Halland	Trönninge[2]	Roadside at Pilsgården	6339171	1289850	35
7	*R. wahlbergii*	Scania	Brunnby	Roadside at Bräcke	6238774	1397980	35

[1,2] Note that there are two Trönninge parishes in Halland. The first ([1]) is south of the city of Halmstad, whereas the second ([2]) is east of the city of Varberg.

Table 2. Number of localities observed for the various forms of *Corylifolii* in the province of Halland.

Figure 1. *R. vikensis* from Onsala (top) and Trönninge (bottom).

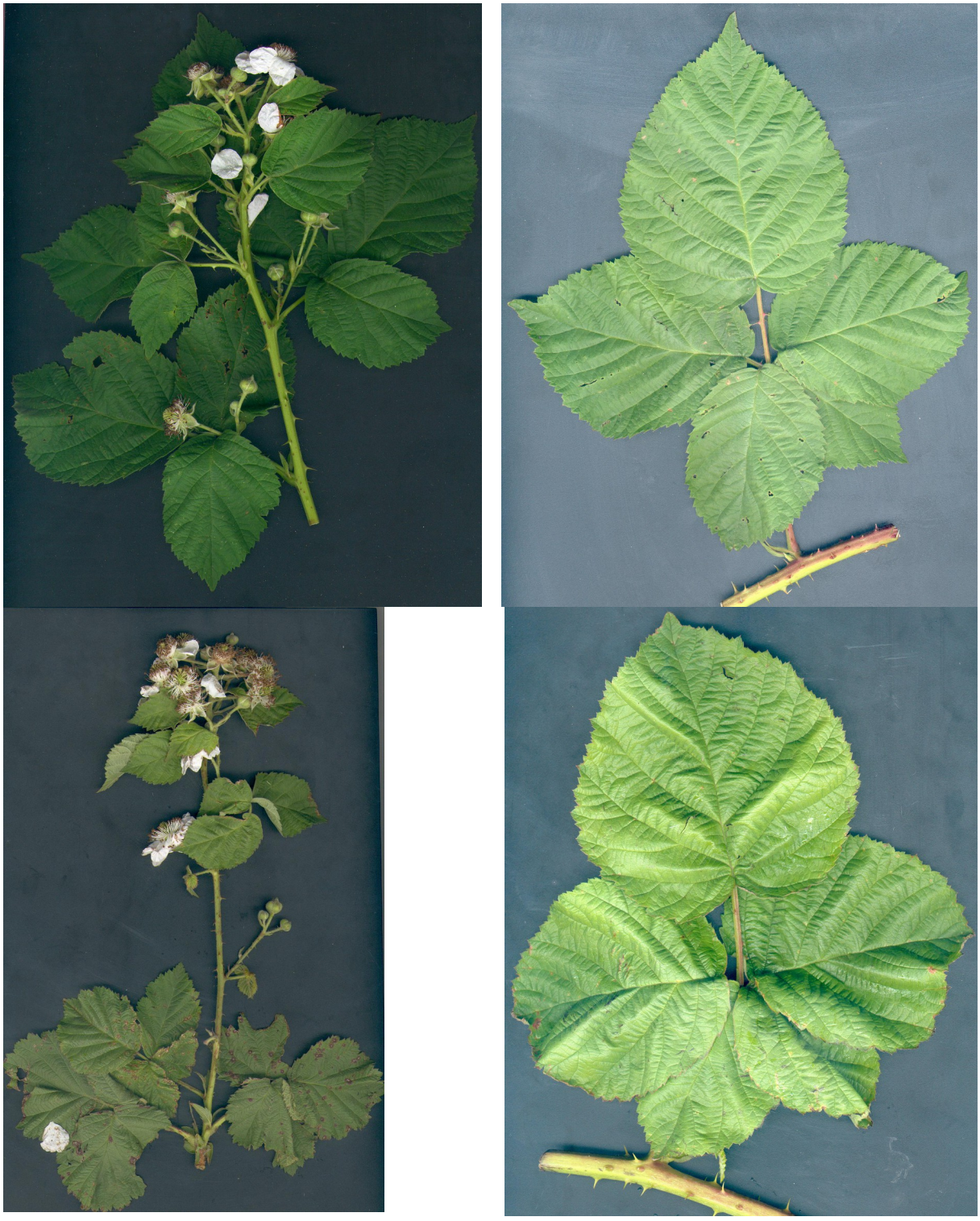


Figure 2. MDS presentation of the RAPD analysis. The numbers of the samples correspond to those in Table 1 (note that samples 3 and 4 almost overlap, as do the two samples from locality 2).

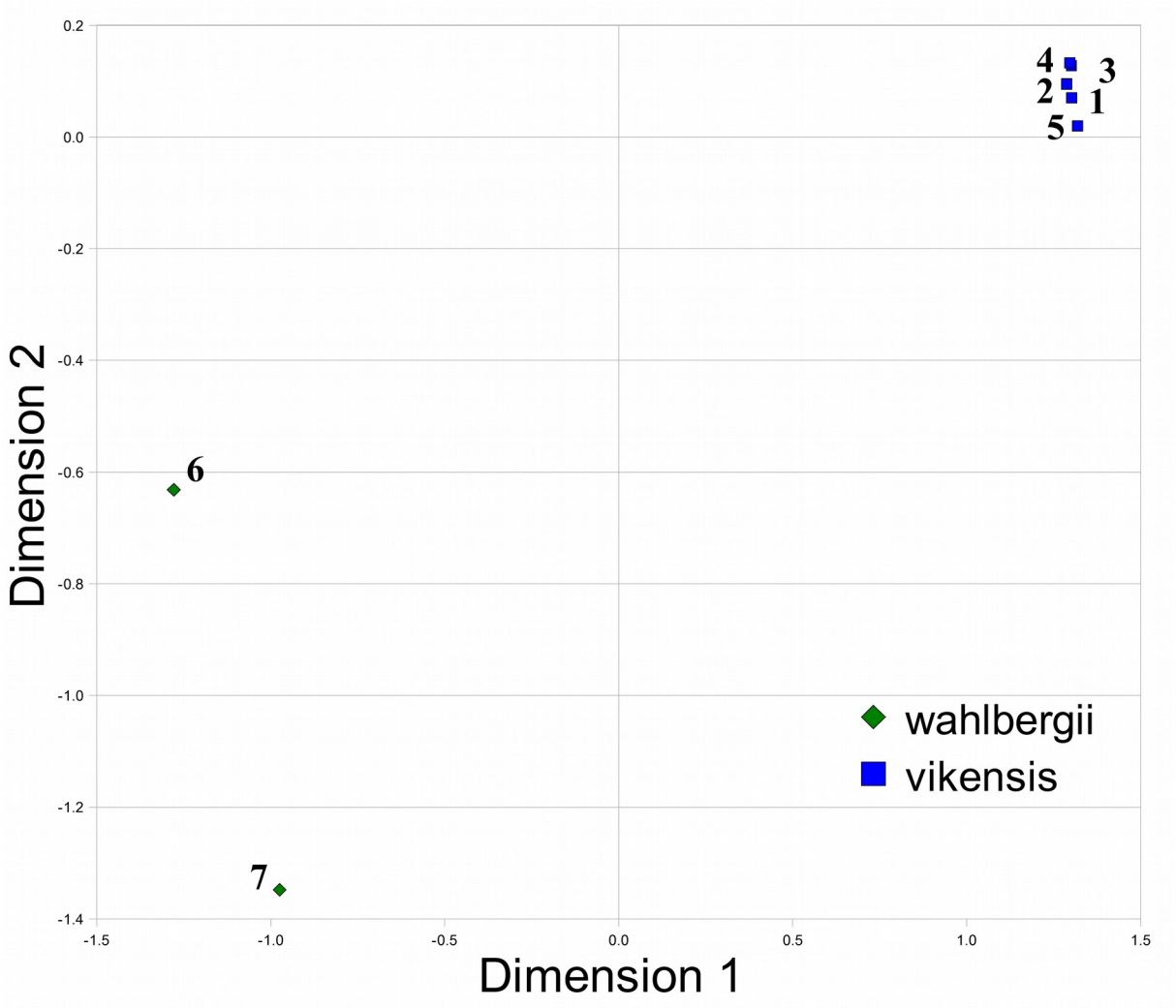
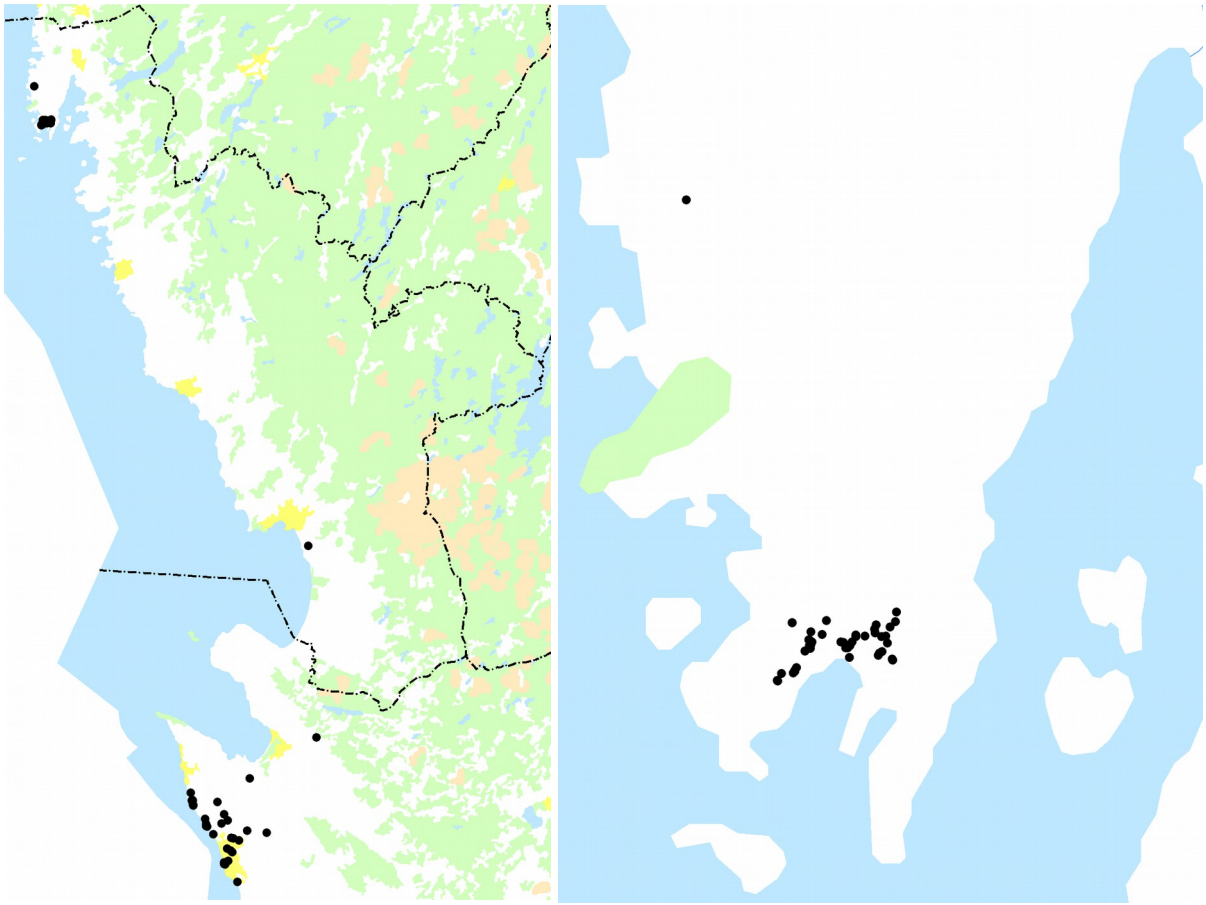


Figure 3. The total distribution of *R. vikensis* in Sweden (left) and on Onsala peninsula (right).



Appendix. A list of known localities of *R. vikensis* in the province of Halland. Coordinates are GPS Swedish Grid RT90. All localities were visited 2000–2008.

Onsala parish
S roadside at Knastås, 6366696 1269100; roadside of Svartakärrsvägen, 636680 1269168; W roadside at Knastås, 6366814 1269363; 6366843 1269386; W roadside at Knastås, 6366855 1269391; W roadside at Knastås, 6366898 1269415; S roadside at Vässingsö, 6367026 1270987; N roadside at Vässingsö, 6367041 1270979; NW roadside at Röda Holmen, 6367069 1270283; S roadside at Vässingsö, 6367102 1270750; W roadside at Vässingsö, 6367142 1270773; E roadside at Vässingsö, 6367161 1270814; W roadside at Knastås, 6367169 1269551; Knastås, 6367169 1269552; path at Knastås, 6367221 1269638; N corner of S Bockskärsvägen in Röda Holmen, 6367229 1270253; W roadside in Röda Holmen, 6367230 1270224; Knastås, 525 m SSO Häckelhagen, 6367261 1269623; S Bockskärsvägen in Röda Holmen, 6367264 1270293; path at Knastås, 6367282 1269655; Knastås, 6367302 1270899; Malövägen in Röda Holmen, 6367314 1270187; path at, Knastås, 6367316 1269651; the crossing between S Bockskärsvägen and Lingonstigen in Röda Holmen, 6367319 1270324; Knastås 6367322 1269667; Malövägen in Röda Holmen, at the post boxes, 6367324 1270141; Knastås at the house, 6367360 1269614; Knastås, 525 m SSO Häcklehagen, 6367361 1269623; S Bockskärsvägen, Röda Holmen, 6367408 1270385; the way to Abbelås, 6367413 1270803; the crossing between Godhemvägen and Vässingsövägen, 6367415 1270872; NW roadside in Röda Holmen, 6367415 1270535; the crossing between S Bockskärsvägen and Ada Karlssons väg in Röda Holmen 6367438 1270389; roadside at Knastås, 6367446 1269833; Runås, 6367473 1270700; Knastås, 525 m SSO Häcklehagen, 6367490 1269650; S roadside at Runås, 6367529 1270692; Several 100 m long stand along the road passing Godhem , 6367570 1270947; roadside at Runås, 6367606 1270720; Runås, 6367636 1269347; the corner at Klockfoten, 6367636 1269345; roadside at Godhem, 6367654 1271035, 2003-06-29; Runås in the forest, 6367676 1269902; W roadside, N of Godhem, 6367814 1271049.

Vallda parish
The NV corner of the crossing of Hästakärrsvägen and Smarholmsvägen, 6374559 1267609.

Trönninge parish
Marl pit, 450 m SW of Trönninge church, 6280569 1323531.